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Cellular Immunity in Chronic Chagas' Disease

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The cellular immune response was assessed in 20 patients with chronic Chagas' disease (American trypanosomiasis). Thymus-derived lymphocyte function was determined in vivo by cutaneous reactivity to several antigens including a soluble preparation derived from *Trypanosoma cruzi* and sensitization to 2,4-dinitrochlorobenzene. The in vitro T-cell reactivity was investigated by the proliferative response to phytohemagglutinin and to *T. cruzi* antigen and by inhibition of leukocyte migration with the specific antigen. In addition, the proportion and absolute numbers of peripheral blood T- and B-lymphocytes were determined by rosette formation. This research indicates that the general and specific cellular immune response, evaluated by the tests herein mentioned, is well preserved in patients, with Chagas' disease. We conclude that chronic Chagas' disease is not associated with deficiency in cellular immunity, nor does it lead to it. Conceivably, the active participation of delayed hypersensitivity may play an important role in the expression of the human chagasic lesions.

Chagas' disease, caused by *Trypanosoma cruzi*, is endemic to a large area of Central and South America. Individuals with chronic infection frequently develop severe myocardiopathy and, sometimes, esophageal and intestinal lesions. The nature of the acquired protective immunity in *T. cruzi* infections, as well as the pathogenesis of the lesions, are not clearly understood. Antibodies against *T. cruzi* have been demonstrated by the complement fixation test, immunofluorescence, and hemagglutination (2, 23, 25).

However, the role played by antibodies in protective immunity is controversial (6, 22). On the other hand, there is experimental evidence supporting the participation of cell-mediated immunity in the host resistance (7, 16, 18, 20). Cellular immunity has been only partially studied in patients with Chagas' disease. Cutaneous tests with *T. cruzi* antigen have been reported in a small number of patients (23, 26). A few in vitro investigations were done by studying the lymphocyte proliferative response to phytohemagglutinin (PHA) and specific antigen using morphological methods (23), and the inhibition of leukocyte migration (8, 25). This report presents a comprehensive assessment of cellular immunity, both in vivo and in vitro, in patients with chronic Chagas' disease.

MATERIALS AND METHODS

Twenty patients with the diagnosis of chronic Chagas' disease were studied. There were 11 males and 9 females, ranging in age from 19 to 68 years. Seventeen patients were found to have myocardial lesions, whereas only three had detectable intestinal lesions. Thirty normal subjects, from the same geographic area, matched by sex and age, were used as the control for cutaneous tests. Twenty of them served as leukocyte donors for in vitro tests.

A soluble antigen was prepared from cultures of the "Y" strain of *T. cruzi* (21) maintained in diphasic medium (5) and harvested after 7 days of culture. The cultures were filtered, and the parasites were washed and suspended in 0.85% NaCl at a concentration of 10^7 cells per ml. This suspension was subjected to sonic treatment in a Biosonik apparatus, and the disintegration of the microorganisms was confirmed microscopically. After centrifugation for 20 min at 12,000 × *g* and 4°C, the supernatant was diluted to a concentration of 30 μg of protein per ml in 0.85% NaCl for the in vitro test and to 20 μg of protein per ml in Coca solution (1:5) for use in cutaneous tests. These concentrations were established according to other authors (8, 23) and proved to be adequate. Both preparations were sterilized by membrane filtration (Millipore Corp.), samples were separated for sterility tests, and the antigen was stored at −20°C in small samples.

Sensitization with 2,4-dinitrochlorobenzene (DNCB), as well as intracutaneous tests, were performed by methods previously described (3, 9). Skin reactions consisting of erythema and induration at 48 h, with or without vesiculation or bullae, were accepted as evidence of sensitization to DNCB. Delayed cutaneous hypersensitivity skin tests were accomplished by intradermal injection of 0.1 ml of the following antigens: purified protein derivative, 2 TU/0.1 ml (Statensseruminstitut, Copenhagen,

Denmark); trichophytin, 1:1,000 (Institute Pasteur, Paris, France); oidiomycin, 1:100 (Allergy Division of Hospital das Clínicas, São Paulo, Brazil); PHAc, 20 μg/ml (purified PHA for clinical use; Wellcome Reagents, England); and *T. cruzi* antigen, 20 μg of protein per ml. The arithmetic mean of the two largest diameters of the area of induration was calculated in millimeters at 48 h for antigens and at 24 h for PHAc. Nodules equal to or larger than 5 mm were considered positive delayed hypersensitivity reactions. Biopsies were taken from the positive reaction to *T. cruzi* for histological examination.

The proportion and absolute numbers of peripheral blood T- and B-lymphocytes were determined by rosette formation with, respectively, sheep erythrocytes (E) and human erythrocytes sensitized with antibody and complement (HEAC), according to methods described in detail elsewhere (10, 13).

Lymphocyte cultures were performed by methods used in previous studies (12, 15). Briefly, peripheral heparinized blood was obtained from patients and normal control subjects. After separation of leukocyte-rich plasma, the lymphocyte concentration was adjusted to 0.3×10^6/ml of Eagle minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) containing 20% autologous or homologous plasma. Each culture tube received 2.5 ml of this suspension, and triplicates were prepared, receiving 0.10 ml of PHA-P (Difco, 1:100 dilution) or *T. cruzi* antigen adjusted to a concentration of 3 μg of protein per ml. Control tubes did not receive any stimulus. Tritiated thymidine, 2 μCi (specific activity, 2.0 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to each tube 6 h prior to termination of cultures. Cultures stimulated with PHA were harvested at 72 h, and those containing specific antigen were harvested at 120 h. [^{3}H]thymidine uptake was determined in a Beckman scintillation counter. The results are expressed as the "stimulation index" (SI), which is the ratio: counts per minute of stimulated tubes/counts per minute of control tubes.

Inhibition of peripheral leukocyte migration was done according to the technique previously described (15). Blood was taken with 20 U of preservative-free heparin per ml and allowed to sediment at 37°C for 1 h. The leukocyte-rich plasma was withdrawn and centrifuged at $200 \times g$ for 10 min. The pellet was washed twice in Hanks balanced salt solution, and the cells were resuspended in Eagle medium containing 10% normal homologous serum at 3×10^7 leukocytes per ml. A polyethylene capillary tube was filled with the leukocyte suspension, cut into segments of 5 cm in length, and sealed at one end with wax. The capillaries were attached to the outer face of a test tube and centrifuged at $150 \times g$ for 10 min. The capillary tubes were cut at the cell-fluid interface, and two capillaries per culture chamber were mounted on silicone dabs, with the open end directed to the center of the chamber. The control chambers were filled with 1.5 ml of Eagle medium containing 10% human serum alone. Test chambers received, in addition, *T. cruzi* antigen at a concentration of 6 μg of protein per ml. Culture chambers were sealed with a cover slip and incubated for 18 h at 37°C. After incubation, the migration area was mapped by projection microscopy and the internal area (the dense halo around the capillary tube) was measured. The results are expressed as the "migration index," which is the ratio: (migration area with antigen/migration area without antigen) × 100.

RESULTS

The percentage of positive delayed cutaneous reactions and the mean values of lymphocyte counts, proliferative response of lymphocytes, and leukocyte migration are summarized in Table 1.

When tested with the antigen extracted from the etiological agent, all patients presented positive delayed cutaneous reactions, ranging from 8 to 30 mm (mean, 12.3 mm). The histology of the reactions to *T. cruzi* antigen showed a mononuclear infiltrate with predominance of lymphocytes. Among normal subjects from the same geographic area, the percentage of positivity was 20%; reactions ranged from 5 to 8 mm, with a mean of 6.4 mm. The difference

TABLE 1. *Percentage of positive delayed cutaneous reactions, mean values of T- and B-lymphocytes in peripheral blood, and proliferative response of lymphocytes and leukocyte migration from 20 patients with chronic Chagas' disease and normal controls*

Determination	Patients	Controls
Cutaneous reaction (%)		
T. cruzi antigen[a]	100	20
Purified protein derivative	40	43
Oidiomycin	80	70
PHAc	100	100
DNCB	90	90
Lymphocyte counts		
Lymphocytes/mm^3	1,764	1,795
T-cells/mm^3	1,131	1,288
B-cells/mm^3	370	382
T-cells (%)	63	62
B-cells (%)	21	24
SI[b]		
PHA, autologous plasma	33.1	41.8
PHA, homologous plasma	41.4	45.3
T. cruzi antigen, autologous plasma[c]	3.1	0.8
T. cruzi antigen, homologous plasma[c]	4.3	0.8
Migration index[c, d]	21.7	88.5

[a] Statistically significant difference by chi-square test ($P < 0.001$).

[b] The SI was calculated as the ratio of thymidine uptake between stimulated and nonstimulated cultures.

[c] Statistically significant by Mann-Whitney test (5% level).

[d] MI = (mean area of migration with *T. cruzi* antigen/mean area of migration without *T. cruzi* antigen) × 100.

between the percentage of positivity to *T. cruzi* antigen in patients and controls was statistically significant by the χ^2 test ($P < 0.001$). The percentage of positivity to the intradermal test with purified protein derivative, oidiomycin, and PHAc, as well as to DNCB sensitization, was similar in patients and normal controls.

The absolute number and percentage of peripheral blood E and HEAC rosette-forming cells observed in patients with chronic Chagas' disease did not differ statistically (Mann-Whitney test) at the 5% level from those of normal controls.

The proliferative response to PHA among patients with Chagas' disease was almost normal. Our experience with a large number of PHA lymphocyte cultures from normal subjects indicated that cultures with SIs lower than 10.0 are depressed and those with SIs between 10.0 and 20.0 are slightly depressed. Lymphocytes from three of the patients showed the proliferative response to PHA to be slightly diminished in autologous plasma, but not in homologous plasma.

We considered a positive proliferative response to *T. cruzi* antigen to be cultures with a stimulation index equal to or greater than 2.0. Fourteen (70%) of the patients showed a proliferative response to the specific antigen of higher than 2.0 in autologous plasma, and 18 (90%) showed this in homologous plasma. This response was significantly higher at the 5% level, by the Mann-Whitney test, than that observed in normal donors. The mean of the response of the control group to *T. cruzi* antigen was 0.8 in autologous and homologous plasma. Only one of the normal subjects presented a positive proliferative response (SI = 1.8) in autologous plasma, but the response in homologous plasma was negative (SI = 1.1).

Migration indexes lower than 75 were considered inhibitory. Peripheral leukocytes of all the patients were inhibited in their migration in the presence of *T. cruzi* antigen. Leukocytes from normal donors were not inhibited. The difference between patients and controls regarding inhibition of leukocyte migration with the specific antigen was highly significant by the Mann-Whitney test.

DISCUSSION

Evaluation of the thymus-dependent immune competence in this group of patients demonstrated that cellular immunity is preserved in chronic Chagas' disease. The percentage and absolute numbers of peripheral T- and B-lymphocytes, the response to cutaneous tests with ubiquitous antigens, the ability to be sensitized by DNCB, and the proliferative response of lymphocytes to PHA did not differ significantly in patients and the control group. The patients' lymphocytes response to PHA in vitro was normal in either homologous or autologous plasma, indicating absence of humoral blocking factors. Unlike that observed in some other infectious diseases (11, 15, 24), American trypanosomiasis does not seem to contribute to depression of the general mechanisms of cellular immunity.

Only among patients with a chagasic megacolon could some degree of impairment of cellular immunity be foreseen. We studied only three of these patients, but it is interesting to note that two of them were unable to be sensitized with DNCB and showed a proliferative response slightly depressed to PHA and *T. cruzi* antigen in autologous plasma. Further work is needed before proposing that patients with intestinal lesions may differ from those with cardiopathy in presenting some impairment of the immune competence.

Concerning the response to *T. cruzi* antigen, our results confirm and extend those described by others (8, 23, 25) in demonstrating that patients with Chagas' disease are able to show delayed hypersensitivity to the etiological agent. With the soluble antigen prepared by us, we observed 100% positivity in delayed cutaneous reactivity among patients compared to 20% in normal subjects from the endemic area. In keeping with the in vivo response, peripheral leukocytes from all the patients were inhibited in their migration in the presence of the *T. cruzi* antigen. Although Lelchuk et al. (8) found that soluble antigens are less effective than particulate antigens in promoting inhibition of leukocyte migration, we were able to find 100% positivity in this test in our patient population, whereas the control group showed no migration inhibition. Patients' lymphocytes showed a proliferative response to *T. cruzi* antigen in autologous and homologous plasma significantly higher than that of the controls. Tschudi et al. (23) obtained similar results using morphological methods to evaluate blastic transformation.

We did not find any evidence that some specific failure in cellular immunity to the parasite could be responsible for the chronicity of Chagas' disease. On the contrary, our results are compatible with the hypothesis that cell-mediated immunity may be operative in the pathogenesis of the lesions.

There is accumulating evidence that a steady balance between host and parasite is usually present in Chagas' disease and that

cellular immunity is important in this balance: parasitemia is very low, and it is remarkably difficult to find parasites at the sites of the lesions (4); outbreaks of high parasitemia can be obtained only by using strong immunosuppressive agents (1, 18); experimental, acute Chagas' disease is easily induced after neonatal thymectomy or treatment with antithymocyte serum (17, 20); and immunity is achieved by lymphocyte transfer (7, 16, 18). On the other hand, the presence in the heart of mononuclear cell infiltrates suggests the possible participation of immune mechanisms, especially autoimmune and delayed hypersensitivity reactions, in the pathogenesis of the chagasic cardiopathy (14). In keeping with these concepts, Santos-Buch and Teixeira (19) have recently reported in vitro lysis of allogeneic, parasitized, and nonparasitized heart cells by *T. cruzi*-sensitized rabbit lymphocytes. The destruction of nonparasitized heart cells and the recognition of a cross-reaction between antigens from parasite and host heart myofibers have indicated that a parasite-induced, cell-mediated immune reaction directed towards the host cell is the basis of subsequent myocardial injury in experimental Chagas' disease.

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